Exhibit A

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Conversion of an Extracellular Dpp/BMP Morphogen Gradient into an Inverse Transcriptional Gradient

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Summary

Morphogen gradients control body pattern by differentially regulating cellular behavior. Here, we analyze the molecular events underlying the primary response to the Dpp/BMP morphogen in *Drosophila*. Throughout development, Dpp transduction causes the graded transcriptional downregulation of the *brinker* (*brk*) gene. We first provide significance for the *brk* expression gradient by showing that different Brk levels repress distinct combinations of wing genes expressed at different distances from Dpp-secreting cells. We then dissect the *brk* regulatory region and identify two separable elements with opposite properties, a constitutive enhancer and a Dpp morphogen-regulated silencer. Furthermore, we present genetic and biochemical evidence that the *brk* silencer serves as a direct target for a protein complex consisting of the Smad homologs Mad/Medea and the zinc finger protein Schnurri. Together, our results provide the molecular framework for a mechanism by which the extracellular Dpp/BMP morphogen establishes a finely tuned, graded read-out of transcriptional repression.

Introduction

It was proposed more than a century ago that the organization of cell and body patterns might be controlled by concentration gradients of "form-producing" substances or morphogens (Morgan, 1897; Turing, 1952; Wolpert, 1989). Only recently has it been possible to demonstrate that secreted proteins of the Wnt, Hedgehog, and transforming growth factor-β (TGFβ) families specify positional information by this mechanism (reviewed by Gurdon and Bourillot, 2001).

Particularly compelling evidence for the existence of an extracellular morphogen gradient comes from studies on the developing wing imaginal disc of *Drosophila*, where a localized source of the BMP2/4 homolog Decapentaplegic (Dpp) is expressed in a stripe of cells along the anteroposterior compartment boundary and exerts a direct and long-range organizing influence on both the anterior and posterior halves (reviewed by Strigini and Cohen, 1999; Podos and Ferguson, 1999). In addition to controlling growth, Dpp induces the expression of different target genes above distinct threshold concentrations. These targets include *vestigial* (*vg*), *optomotor-blind* (*omb*), and *spalt* (*sal*), are expressed in progressively narrower domains, define the primordium of the wing blade, and control important aspects of pattern, differentiation, and survival (Kim et al., 1996; Grimm and Pflugfelder, 1996; Sturtevant et al., 1997; reviewed by Podos and Ferguson, 1999).

An understanding of how morphogen gradients operate requires answers to two different questions. How do concentration gradients arise, and how do cells interpret different morphogen concentrations? While recent efforts in the field focused on the problem of how Dpp protein spreads through tissue (Ramírez-Weber and Kornberg, 1999; Entchev et al., 2000; Teleman and Cohen, 2000), we are here concerned with the question of how a Dpp gradient is converted into transcriptional outputs.

Like all members of the TGFβ superfamily, Dpp assembles at the cell surface a receptor serine/threonine kinase complex comprising subunits known as the type I and type II receptors, encoded by the genes *thick-veins* (*tkv*) and *punt*, respectively (reviewed by Massagué, 1998; Podos and Ferguson, 1999; Tabata, 2001). The binding of Dpp to its receptors triggers the phosphorylation of Tkv by Punt and in turn enables Tkv to recognize and phosphorylate the Smad protein Mad (Raftery and Sutherland, 1999; Tanimoto et al., 2000). Phosphorylation releases Mad from cytoplasmic retention, allowing its association with the related factor Medea (Med) (Hudson et al., 1998; Inoue et al., 1998; Wisotzkey et al., 1998) and subsequent translocation into the nucleus, where the two proteins are involved in the transcriptional regulation of target genes (reviewed by Massagué and Wotton, 2000; Affolter et al., 2001).

Mad and Med possess DNA binding activities that have been implicated in the recognition of a regulatory element in the *vg* gene (Kim et al., 1997). Hence, *Drosophila* Smad proteins have been proposed, in analogy to their vertebrate counterparts, to directly activate the Dpp targets *vg*, *omb*, and *sal*. An alternative mechanism has recently emerged with the unexpected discovery of Brinker (Brk), a transcription factor that is required to counteract responses to Dpp (Campbell and Tomlinson, 1999; Jaźwińska et al., 1999a; Minami et al., 1999). Loss of Brk function causes overproliferation and ligand-independent, ectopic expression of the Dpp targets *vg*, *omb*, and *sal*. *brk* expression itself is negatively regulated by Dpp, such that peripheral cells in the wing disc express high and central cells undetectable levels of Brk. These findings raise the possibility that it is primarily the repressive function of Brk that controls growth and Dpp target gene expression and that direct transcriptional activation by Mad may only play a subordinate role.

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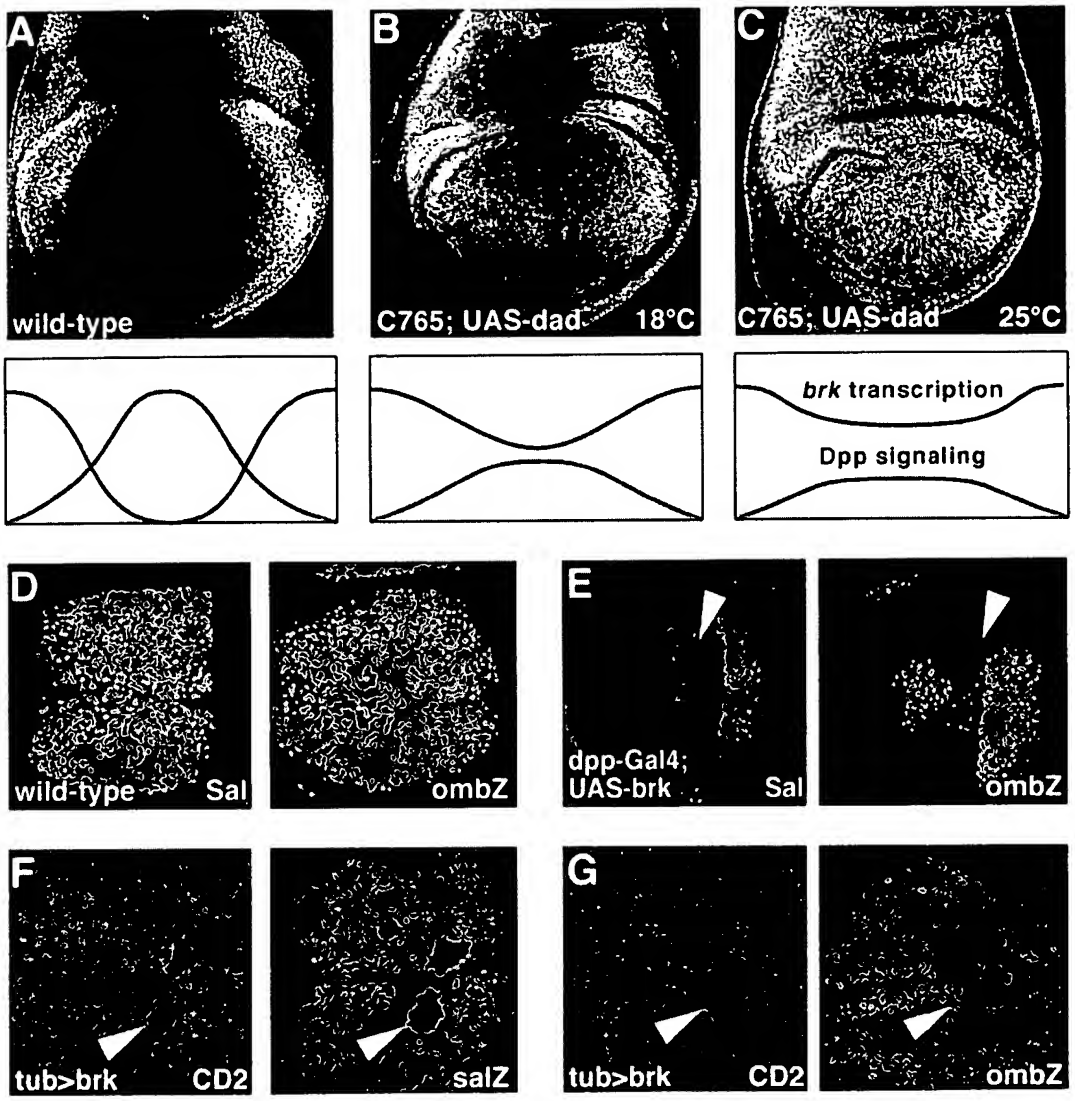


Figure 1. The Dpp Signaling Gradient Determines the Profile of *brk* Expression, Which in Turn Defines the Activity States of Dpp Target Genes

(A–C) Confocal sections of wing discs are shown (dorsal up, anterior to the left). *brk* expression is visualized by means of the *brk-lacZ* reporter *X47* in wild-type (A) and when Dpp signaling is downregulated by expressing the inhibitory Smad *Daughters against dpp* (*Dad*, [B and C]). The low-level ubiquitous *C765-Gal4* line was used to induce a *UAS-dad* transgene, which results in a shallower, and hence better detectable, *brk* gradient. Below each panel our interpretation is shown in the form of diagrams, which indicate the inverse relation between Dpp signaling levels (red) and *brk* expression levels (blue).

(D–G) Different Brk levels define distinct combinations of target gene expression. (D) Wild-type expression patterns of the Dpp target genes *sal* (in green) and *omb* (in red) are shown in wing primordia. (E) High levels of ectopic Brk expression were obtained with *dpp-Gal4 UAS-brk*. These levels of Brk repress the expression of both *sal* (E, left) and *omb* (E, right). The domain of *dpp-Gal4* activity is broader than the domain of endogenous *dpp* expression, hence the widespread effect in the anterior compartment. *dpp-Gal4*, rather than *actin5c>Gal4*-expressing clones, was used in this experiment to drive *UAS-brk* expression, because clones ectopically expressing substantial levels of *brk* rapidly undergo apoptosis in the wing pouch epithelium. (F and G) Low levels of ectopic Brk were obtained with a *tubulinα1>CD2>brk* construct. Clones expressing *brk* under the *tubulinα1* promoter (*tub>brk*) are marked by the absence of CD2 staining (in green). In such clones, *sal* expression is repressed (F), but *omb* expression is unaffected (G).

Here, we provide strong support for this view by studying the role and establishment of the Brk gradient. We find that the output of Dpp signaling and the action of the zinc finger protein Schnurri (Shn), both of which have been implicated by genetic means in the regulation of *brk* (Marty et al., 2000; Torres-Vazquez et al., 2000), converge on defined silencer elements of *brk*. The resulting inverse expression gradient of nuclear Brk protein has the capacity to differentially regulate *omb* and *sal*. Our results provide the molecular framework for a mechanism in which the extracellular Dpp gradient is converted into primary nuclear outputs via the generation of an inverse transcriptional gradient of *brk* by means of Shn-dependent silencer elements.

Results

Dpp Signaling Levels Control the Profile of the Brk Expression Gradient

High levels of Dpp signaling prevent the expression of the *brk* gene (Campbell and Tomlinson, 1999; Minami et al., 1999). In contrast, *brk* is readily transcribed in cells situated far away from a Dpp source or in cells with an experimental block in the Dpp transduction pathway (Campbell and Tomlinson, 1999; Jaźwińska et al., 1999a; Minami et al., 1999). In leg and wing imaginal discs, lateral cells, expressing maximal levels of *brk*, and central cells, in which *brk* expression cannot be detected, are separated by a seemingly narrow stripe of cells with graded *brk* expression. To explore whether position and spatial extent of this population are sensitive to Dpp signaling levels, we altered the presumptive Dpp signaling gradient by ubiquitously expressing the inhibitory Smad6 homolog Dad (Tsuneizumi et al., 1997). Low Dad levels cause a significant expansion of the *brk*-expressing domains toward the center of the disc (Figure 1B, *C765-Gal4 UAS-dad* at 18°C) with an extended, shallow gradient of *brk* levels. Higher levels of Dad (Figure 1C, same genotype at 25°C) produce an even more pronounced effect with cells along the entire anteroposterior (AP) axis expressing *brk*. We interpret these observations as indication that different levels of Dpp signaling determine, with an inverse relationship, different levels of *brk* expression. These experiments taken together with the genetic requirement of *brk* for regulating target genes (data not shown) suggest that the functional Brk gradient extends beyond the domain in which graded *brk* expression can be detected with reporter genes in wild-type.

Brk Expression Levels Control the Activity States of Dpp Target Genes

The Dpp target genes *vg*, *omb*, and *sal* are expressed in nested domains with progressively narrower widths of activity along the AP axis. The expression of all three of these genes is subject to repression by Brk in lateral regions of the wing disc (Campbell and Tomlinson, 1999; Jaźwińska et al., 1999a; Minami et al., 1999), raising the possibility that different levels of *brk* alone are able to specify distinct combinations of activity states of these genes. To address this possibility, we asked whether low levels of ectopic Brk expression can repress *sal*, but not *omb*, transcription, whereas high levels of Brk levels would repress both genes. High levels of ectopic Brk expression in the center of the disc were obtained by using a *UAS-brk* transgene in conjunction with a *dpp-Gal4* driver. Low levels of Brk were expressed by the weak constitutive promoter from the *tubulinα1* gene in marked clones of cells. As shown in Figure 1E, the *dpp-Gal4 UAS-brk* transgenes cause repression of both *sal* and *omb* transcription. In contrast, the lower levels of *brk* produced by *tubulinα1>brk* repress only *sal*, while *omb* transcription is not affected. Hence, different levels of Brk expression can elicit distinct outputs.

Together, the experiments described so far imply that the transcriptional control of *brk* is a key event in the interpretation of the Dpp morphogen gradient. In order to understand how this morphogen gradient becomes translated into different cell fates, we focused on the question of how Dpp generates an inverse transcriptional gradient of *brk* expression.

Dissection of the *brk* Regulatory Regions into Separable Enhancer and Repression Activities

Our first efforts were directed toward isolating the regulatory elements of the *brk* gene that ensure proper expression levels along the AP axis in response to Dpp signaling. We scanned the 20 kb region between the *brk* transcription unit and its upstream neighboring locus for such elements (Figure 2A). Restriction fragments from genomic *lambda* phages were cloned into a *lacZ* reporter P element and assayed for regulatory activity in vivo. This led to the identification of fragment B14, which faithfully recapitulates all aspects of late embryonic and larval *brk* expression (Figure 2A).

Interestingly, we found that distal truncations of B14 caused a progressive widening of the lateral expression domains toward the center of wing imaginal discs, while the levels of expression remained constant (Figure 2A). This observation suggested to us that the *brk* enhancer consists of two separable entities, a ubiquitously active, constitutive enhancer element located in the proximal half and a regulated repression activity encoded by the distal half.

Both activities were narrowed down by an extensive series of reporter constructs, a small subset of which is shown in Figures 2B–2D (for details, see legend to Figure 2). Three short fragments (called A, B, and C) were identified that possess repression activities when coupled to the constitutive enhancer represented by construct B38 (Figures 2B and 2C). The most potent of these short elements, fragment C, was further dissected into a 53 bp element (Figure 2D), referred to as S (S for *silencer*, see below). Its repression function is encoded in a nonredundant manner, as point mutations abolish its activity (Figure 2D and Experimental Procedures).

The dissection of 20 kb of potential regulatory sequences into two discrete minimal elements with opposite activities, which together reconstitute the hallmarks of *brk* expression, establishes the basis for our molecular studies. As described below, fragment C and its shorter derivative S serve as a paradigm to study the regulation of *brk* repression.

A Signaling-Regulated Silencer: The *brk* Repression Element Can Operate Independently of the *brk* Enhancer, but Its Activity Depends Strictly on Dpp Input

The activities of the *brk* regulatory elements were analyzed in diverse imaginal and embryonic tissues (Figure 3). Invariably, repression activity was maximal in vicinity of well-characterized sources of Dpp, suggesting that this activity is dependent on Dpp signaling. To confirm this apparent requirement for Dpp input, the repression activity was monitored in wing disc cells lacking the Dpp type I receptor Thick-veins (Tkv). *tkv* mutant cells autonomously lost repression activity (data not shown, but see below), indicating that this repression is strictly regulated by Dpp signaling.

Reporter constructs exhibiting spatially decreased domains of repression can therefore be regarded as less

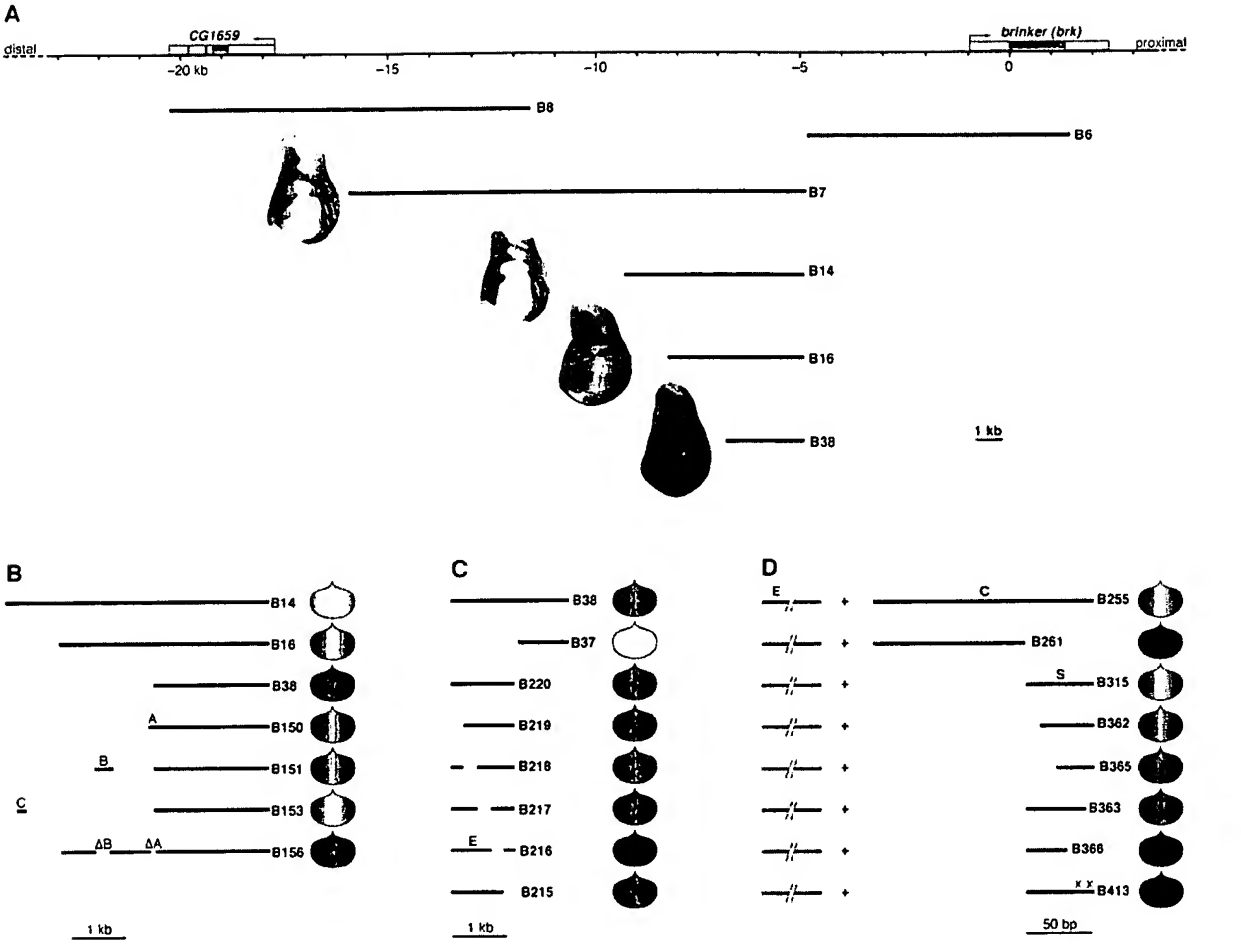


Figure 2. Dissection of the *brk* Regulatory Region into Separable Activating and Repressing Activities

(A) A map of the upstream region of the *brk* locus is shown on top. Restriction fragments B6, B7, and B8 were tested for their ability to drive reporter gene expression in transgenic animals. Fragment B8 did not cause any detectable expression, and no transgenic animals were obtained from fragment B6, likely due to toxicity. Fragment B7 faithfully recapitulated all aspects of *brk* expression and was further reduced in size, leading to the 5 kb fragment B14 that still drives *brk*-like expression. Distal truncations of fragment B14 resulted in a progressive widening of the lateral expression domains in imaginal discs (see B14, B16, and B38), suggesting that B14 contains repression elements in its distal part and a constitutively active enhancer in its proximal part (represented by fragment B38). In all panels, only a small subset of the constructs tested are shown.

(B) Three fragments A, B and C were identified in the distal part of B14 to cause repression in central regions of the wing disc (in combination with the constitutively active enhancer B38), as shown in constructs B150, B151, and B153. Among the three fragments, C showed strongest activity. If sequences A and B are removed from B16 (i.e., construct B156) repression activity is almost completely lost and expression is like that of B38.

(C) Fragment B38 still shows slightly reduced expression in the center of the disc where Dpp signaling is highest. In an attempt to obtain an enhancer fragment that is uniformly expressed, B38 was further dissected. This lead to the identification of B216 (shown in green) that is evenly and ubiquitously expressed in the wing pouch and therefore provides a sensitive tool to test other fragments for their ability to mediate regulated repression. We call B216 "E" for *enhancer*.

(D) Fragment C (see Figure 2B) was chosen for further analysis. The repressive activity of C and of its derived subfragments was assayed in combination with E (Figure 2C), and was localized to a 53 bp subfragment, referred to as "S" (for *silencer*, shown in red). The activity of S was strongly reduced and became unstable by further terminal deletions (B362, B365, B363, and B366). In addition, systematic point mutations throughout S identified base pairs that are essential for repression activity (exemplified by B413), leading us to conclude that S represents a minimal fragment. For nucleotide sequences of S and B413, see Experimental Procedures.

sensitive toward Dpp input. In all tissues examined, the decrease in sensitivity of such reporters is similar to that observed in wing discs (Figure 3), indicating that the *brk* repression element operates throughout embryonic and imaginal stages to perceive the activity state of the Dpp signal transduction pathway.

So far, the *brk* repression element has only been assayed in the context of the constitutive *brk* enhancer, which is part of the same regulatory region in the *Drosophila* genome. We sought to test whether this negative regulatory element can impose Dpp-dependent repression on heterologous enhancers. Below, we use three diverse enhancers in three different systems to provide evidence that this is indeed the case.

First, we used a previously characterized regulatory element of the *dpp* locus, which directs uniform expression within the pouch region of wing imaginal discs (Müller and Basler, 2000). When the *brk* repression ele-

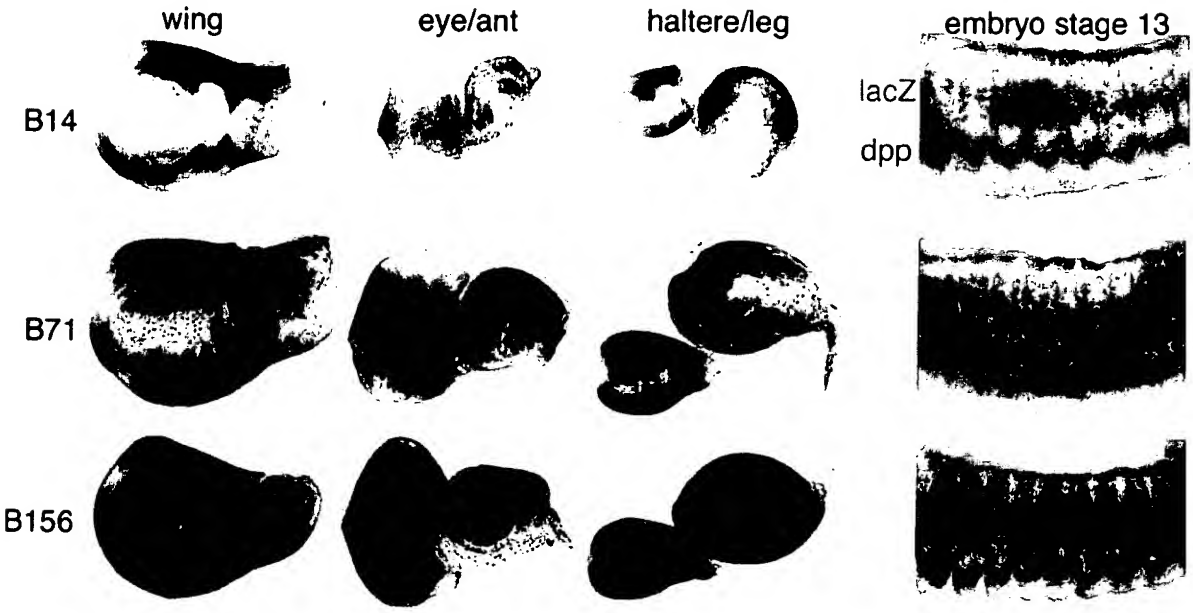


Figure 3. Throughout Embryonic and Larval Tissues, the Activity State of the Dpp Signaling System Is Integrated by the *brk* Regulatory Region

Expression of *brk* reporter constructs with different sensitivities to Dpp signaling are shown: wild-type sensitivity (B14, top row), decreased sensitivity (B71, middle row), and almost absent sensitivity (B156, lowest row). In all larval tissues analyzed (i.e., eye, antennal (ant), haltere, and leg imaginal discs), as well as in mid- and late-embryonic tissues, the sensitivity of the reporter constructs to Dpp is similar to that observed in wing discs, judged by the gap between the expression domains of *dpp* (data not shown for discs) and the reporters. In the embryo (the rightmost panels) *dpp* mRNA expression is shown in blue, and the antibody staining in brown detects the *lacZ* expression of the reporter constructs. Wing discs are oriented with their anterior side up and dorsal to the right.

ment is linked to this enhancer, transcriptional activity is confined to the lateral edges of the wing pouch (Figure 4A). Second, we assayed the embryonic *even-skipped-stripe-2* enhancer (Small et al., 1992) in isolation of, and combination with, the *brk* repression element. This enhancer is normally active in a circumferential band of cells in the early blastoderm stage. However, when linked to *brk* repression elements, the *even-skipped-stripe-2* enhancer is repressed in dorsal regions of the embryo (Figure 4B), where cells are exposed to high levels of Dpp (Ferguson and Anderson, 1992). Finally, we assayed the activity of the *brk* repression element in the context of a Notch-responsive enhancer in *Drosophila* tissue-culture cells. This synthetic enhancer shows a 30- to 40-fold stimulation of reporter gene expression upon transfection of S2 cells with plasmids driving the expression of Suppressor of Hairless (Su[H]) and a constitutively active form of Notch (Kirkpatrick et al., 2001). Simultaneous cotransfection of a plasmid encoding the activated form of the Dpp receptor Tkv (Tkv^{QD}, see Nellen et al., 1996) blocks this activation, in a manner strictly dependent on the presence of the *brk* repression fragment (Figure 4C). Thus, S2 cells are capable of transducing Tkv input and converting it into transcriptional regulation. The repression mediated by the *brk* element in this system occurs in the context of a heterologous enhancer located on transfected plasmid DNA.

Together with our finding that the *brk* repression element can mediate Dpp-dependent repression independently of its orientation or position (data not shown), the above-described results allow us to call this element a "signaling-regulated silencer."

The Net Balance of Silencer and Enhancer Activities Determines the *brk* Expression Levels

The results presented so far indicate that the levels of *brk* expression determine the fate of wing cells along the AP axis and that these levels are defined by three parameters: (1) the degree of activation of the Dpp transduction pathway, (2) the "strength" of the constitutive *brk* enhancer, and (3) the repressive activity of the *brk* silencer at any given degree of Dpp signaling. This model raises the prediction that altering any of the three parameters, while leaving the other two fixed, should have a direct impact on the spatial profile of *brk* expression. In the first result section, we have tested the effects of altering the Dpp signaling levels. We next set out to alter the activity of the *brk* silencer (S). As shown in Figure 5, an increase of its copy number results in a progressive lateral shift of those cells that express high detectable levels of reporter gene activity in the wing disc. Conversely, the duplication of the constitutive *brk* enhancer (E) has the opposite effect and leads to an expansion of reporter gene expression toward the disc center at a given number of *brk* silencer elements. Hence, it is the net balance of the two opposing regulatory forces that determines the level of *brk* expression at any given level of Dpp signaling.

brk Silencer Activity Depends on Mad, Med, and Schnurri Function

Over the past few years, a fairly detailed picture has emerged of how target genes are activated in response to ligands of the TGFβ, BMP, and Activin families in a stage- and tissue-specific manner (reviewed by Mas-

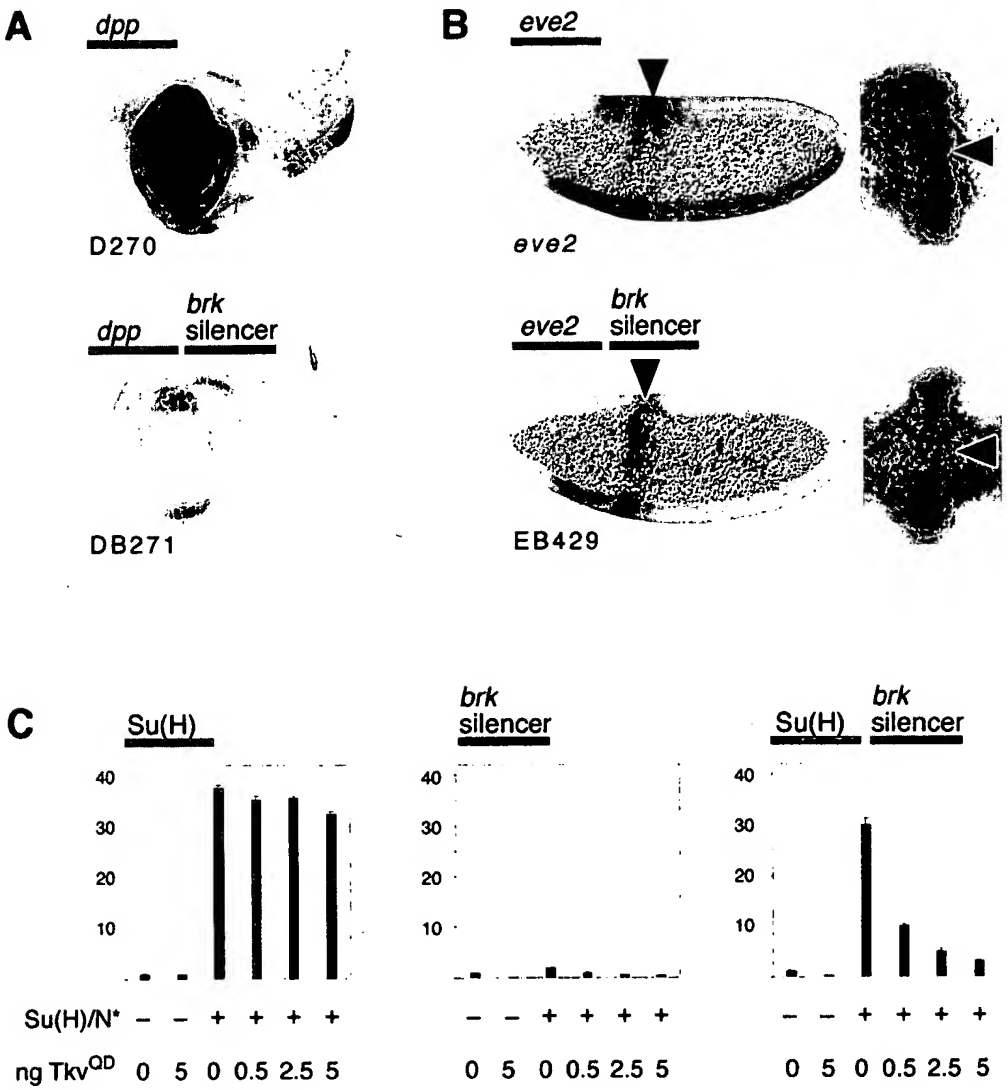


Figure 4. The *brk* Silencer Imposes Repression on Heterologous Enhancers Active in Discs, in the Early Embryo, and in S2 Cells

(A) Construct D270 is derived from a minimal *dpp* enhancer that lacks repressive inputs of both Ci and Engrailed (see Figure 2 of Müller and Basler, 2000) and drives reporter expression in the entire wing pouch. The addition of *brk* silencer fragments in construct DB271 leads to a repression in central domains where Dpp signaling occurs.

(B) A blastoderm-stage embryo in which reporter gene expression is driven by the *even-skipped-stripe-2* enhancer (*eve2*, Small et al., 1992) is shown on top, either in a lateral view (to the left) or dorsal view (to the right, higher magnification). The *eve2* enhancer is fully active on the dorsal side of the embryo (arrowheads). The addition of *brk* silencer elements (construct EB429) causes a repression in dorsal domains where Dpp signaling is highest (arrowheads). The anterior sides of embryos are oriented to the left.

(C) β-galactosidase reporter assays in *Drosophila* S2 cells. Reporter plasmids contain the *lacZ* gene under the control of a Suppressor of Hairless response element (Su[H], in green, left), a fragment of the *brk* control region containing the subfragement S (*brk* silencer, in red, middle), or the combination of the two elements (right). These reporters were cotransfected with a combination of plasmids encoding Su(H) and an activated form of Notch (N*). Increasing amounts of a plasmid expressing Tkv^{QD} lead to a stepwise repression of reporter activity (right). Tkv^{QD}-mediated repression is strictly dependent on the presence of the *brk* silencer since it is not observed with the reporter containing only the Su(H) response element (left). β-galactosidase values were normalized by cotransfecting 5 ng of a plasmid expressing luciferase as an internal standard. Results shown represent the average β-galactosidase activities from transfections done in triplicates (± standard deviation) and are expressed as the X-fold activation over the basal activity of each reporter plasmid alone.

sagué and Wotton, 2000; Attisano and Wrana, 2002). To explore how input by the BMP homolog Dpp causes repression rather than activation of *brk* transcription, and how it can do so in virtually all cells of an organism, we set out to analyze this process by genetic and biochemical means. We first assayed the requirements for the known Dpp signal transduction components in the above-described context in which the *brk* silencer represses transcription driven by the heterologous wing blade enhancer from the *dpp* locus. As shown in Figures 6A–6C, wing cells require the activities of the *tkv*, *Mad*, and *Med* genes to repress reporter gene expression. In addition, the *brk* silencer also depends on Shn function, as *shn* mutant cells ectopically express high levels of

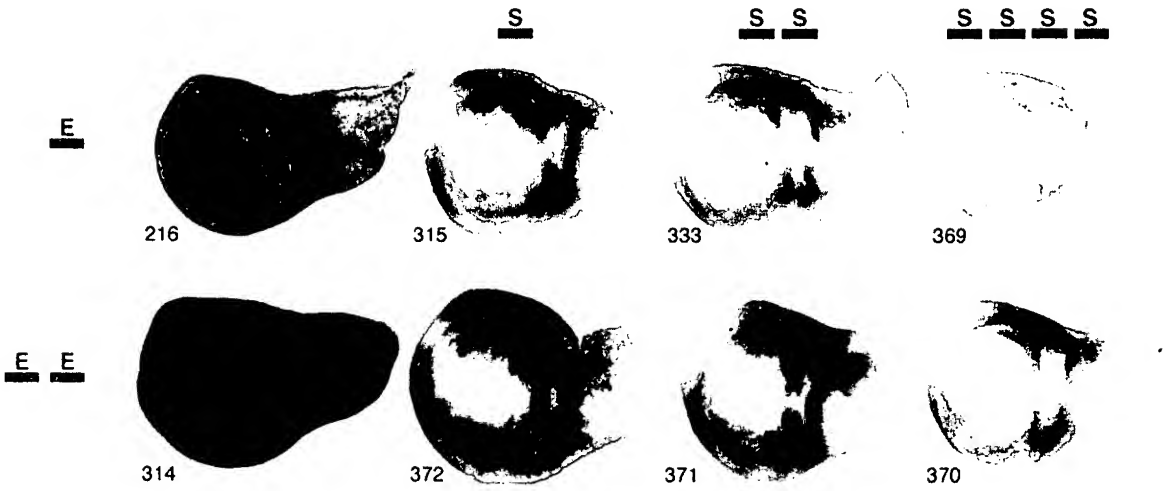


Figure 5. The Net Balance of *brk* Enhancer and Silencer Activities Determines the Transcriptional Output Levels in a Given Cell

None, one, two, or four copies of *brk* silencer elements (S, shown in red) were combined with one (top row) or two (bottom row) *brk* enhancer elements (E, shown in green). An increase in the copy number of S results in a progressive lateral shift of those cells that express high detectable levels of reporter gene activity (both rows, from left to right). Conversely, the duplication of enhancer element E has the opposite effect and leads to an expansion of reporter gene expression at a given number of *brk* silencer elements (compare top row with bottom row). Discs are oriented with their anterior side up.

the reporter gene (Figure 6D). The same requirements were observed in cultured cells, where Tkv^{QD} activity was no longer able to abolish Notch-induced activation of reporter gene expression when either endogenous *Mad* or endogenous *shn* functions were knocked-down by RNAi (Figure 6F). Addition of either double-stranded RNA had no effect, however, on the Notch-stimulated induction in the absence of Dpp signaling.

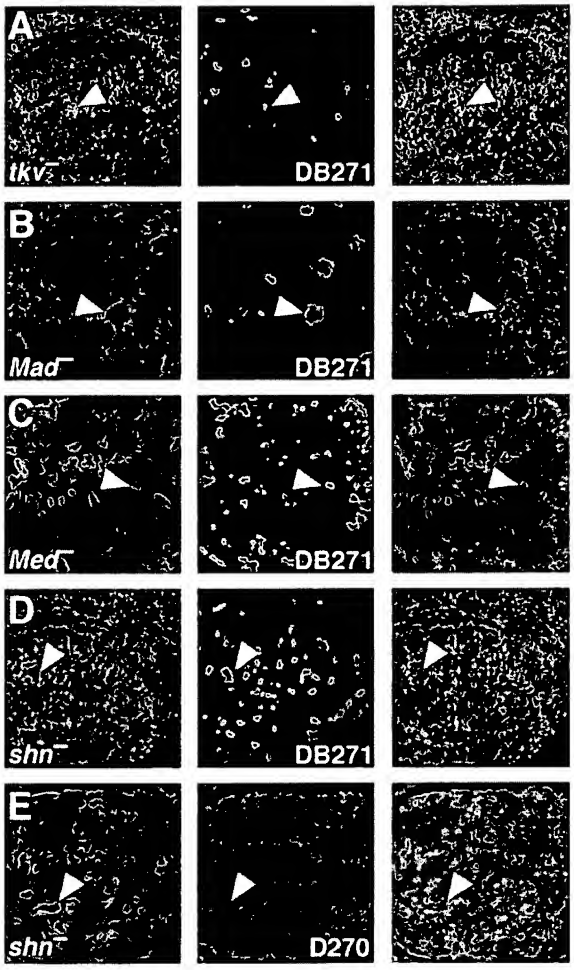
The C-Terminal 640 Amino Acids of Shn Are Necessary and Sufficient for Dpp-Dependent *brk* Repression In Vivo and In Vitro

Our observations that repression by the minimal *brk* silencer shows the same requirements in S2 cells as in vivo (i.e., an activated Dpp receptor, Mad, Med, and Shn) prompted us to analyze this process biochemically with epitope-tagged proteins. However, all our attempts to detect significant amounts of full-length Shn protein in extracts from embryos or from transfected S2 cells failed. The Shn protein is very large (2529 amino acids, Arora et al., 1995; Grieder et al., 1995) and proved to be refractory to biochemical manipulation in our hands. To overcome this limitation, we searched for shorter derivatives of Shn that retained the ability to mediate Dpp-dependent repression of *brk*. In a series of Shn proteins with terminal truncations and/or internal deletions, we identified one short form, referred to as ShnCT, which retained the key properties of full-length Shn. Like transgene-derived full-length Shn, ShnCT is able to repress transcription of the endogenous *brk* gene (data not shown), as well as that of the B14 reporter gene (Figure 7A), in *shn* null mutant embryos in a Dpp-dependent manner. ShnCT comprises the C-terminal 640 amino acids and thus three of the eight Shn zinc finger motifs. In contrast, ShnNT, which comprises all but the 640 residues of ShnCT, or ShnΔZF6-8, which only lacks the three C-terminal zinc fingers, has no detectable rescuing activity (Figure 7A), indicating that these structural motifs play a crucial role in repression via *brk* silencer elements.

To confirm that the same C-terminal Shn sequences are able to mediate *brk* repression in our S2 cell assay, we expressed ShnCT in cells treated with double-stranded RNA against the central portion of endogenous *shn* mRNA and, hence, substituted endogenous Shn protein with ShnCT. Dpp-dependent repression was fully recovered under these conditions (Figure 7B). Furthermore, and as observed in vivo, the three clustered zinc fingers in ShnCT are critical for this rescue of repression, validating our Shn reagent as well as our cell-based transcription assay.

The *brk* Silencer Element Assembles a Shn/Mad/Med Complex

Since all three proteins that are required for Dpp-dependent repression by genetic criteria contain putative DNA binding domains (i.e., Mad, Med, and Shn), we set out to test their ability to molecularly interact with the *brk* silencer element in extracts of Tkv^{QD}-expressing S2 cells. Electrophoretic mobility shift assays indicated that neither ShnCT nor Med was able to form a stable protein/DNA complex (Figure 7C), although both proteins were readily expressed (data not shown). Transfection with a Mad-encoding plasmid resulted in the formation of a detectable protein/DNA complex (Figure 7C, lane 3), but a more prominent complex of similar mobility was obtained upon expression of Mad in combination with Med (Figure 7C, lane 7). Coexpression of ShnCT with Mad and Med led to the formation of a complex of even slower mobility (lane 8), suggesting that ShnCT is recruited to the *brk* silencer element with the help of Mad and Med. In contrast, the complex that formed in the presence



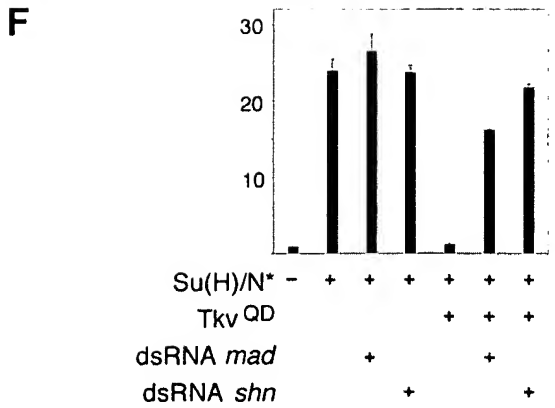


Figure 6. *brk* Silencer Activity Depends on Tkv, Mad, Med, and Shn Function

(A–D) Expression of reporter DB271 (described in Figure 4A) in wing discs with *tkv*, *Mad*, *Med*, and *shn* mutant clones. The left shows the expression of the marker gene (green), the loss of which indicates mutant genotypes. In the middle, the β-galactosidase expression of DB271 is visualized (red). A merge of both images is shown to the right. Expression of DB271 is strongly upregulated in medial *tkv*, *Mad*, and *Med*, as well as in *shn* mutant clones.

(E) In contrast, expression of D270 is not affected by these genotypes. D270 lacks *brk* silencer elements and is expressed ubiquitously in the wing pouch. Representative for the *tkv*, *Mad*, *Med*, and *shn*, only the results for *shn* mutant clones are shown. Arrowheads point to exemplary clones.

(F) *Drosophila* S2 cells were cotransfected with a reporter plasmid containing the *brk* S element fused to the Su(H)-response element, expression plasmids and dsRNA fragments as indicated below the panel. Tkv^{QD}-mediated repression is blocked when endogenous Mad or Shn are "knocked down" by RNAi. β-galactosidase levels are shown as X-fold activation over the basal activity of the reporter plasmid when cotransfected with the empty expression vector. dsRNA fragments are derived from the *Mad* (nucleotides 658–1230) or the *shn* (nucleotides 5011–5531) coding regions.

of Mad and Med was not retarded in its mobility by concomitant expression of the ShnCT variant lacking the three clustered zinc fingers (lane 9).

To investigate the molecular composition of the low mobility protein/DNA complexes, we cotransfected Tkv^{QD}, ^{Flag}Mad, ^{Myc}Med, and ^{V5}ShnCT and assayed for the presence of the Flag, Myc, or V5 epitope tags by supershift analysis upon addition of the appropriate antibodies. In the absence of ShnCT, the complex contained both Mad and Med proteins as evidenced by supershifts with both the anti-Flag and the anti-Myc antibodies (Figure 7D). In the presence of ^{V5}ShnCT, the low mobility complex was additionally supershifted by antibodies directed against the V5 epitope. However, when the same low mobility complex was produced with an untagged version of ShnCT, no increase in mobility was observed upon addition of the anti-V5 antibody, confirming the specificity of the assay (data not shown).

From these biochemical experiments, we conclude that Mad, Med, and Shn form a protein complex on the *brk* silencer element. Nuclear translocation of Mad and Med requires the activation of the Dpp signaling pathway (Raftery and Sutherland, 1999). Since Shn is only recruited to the *brk* silencer element in the presence of Mad and Med, it can be inferred that the complex can only be established in response to Dpp signaling. Moreover, our finding that both transcriptional repression as well as complex formation critically depend on the presence of the C-terminal three zinc finger motifs supports the notion that the *brk* silencer element controls *brk* expression by assembling a Mad/Med/Shn multiprotein complex.

Discussion

Dpp's ability to organize cellular patterns serves as a paradigm for the existence and mode of action of extracellular morphogen gradients. Most notably, Dpp gradients control cell fates along the dorsoventral axis of the early embryo and along the anteroposterior axis of imaginal discs (reviewed by Podos and Ferguson, 1999). In addition to its capacity to act at long range, Dpp elicits distinct outputs at different concentrations (Nellen et al., 1996; Lecuit et al., 1996; Ferguson and Anderson, 1992). BMP activity gradients have also been implicated in the control of vertebrate body pattern, particularly in the establishement of the dorsoventral axes of the early mesoderm, neural tube, and retina (Holley and Ferguson, 1997; Lee and Jessell, 1999; Sakuta et al., 2001). Major interest is devoted, therefore, to the mechanisms

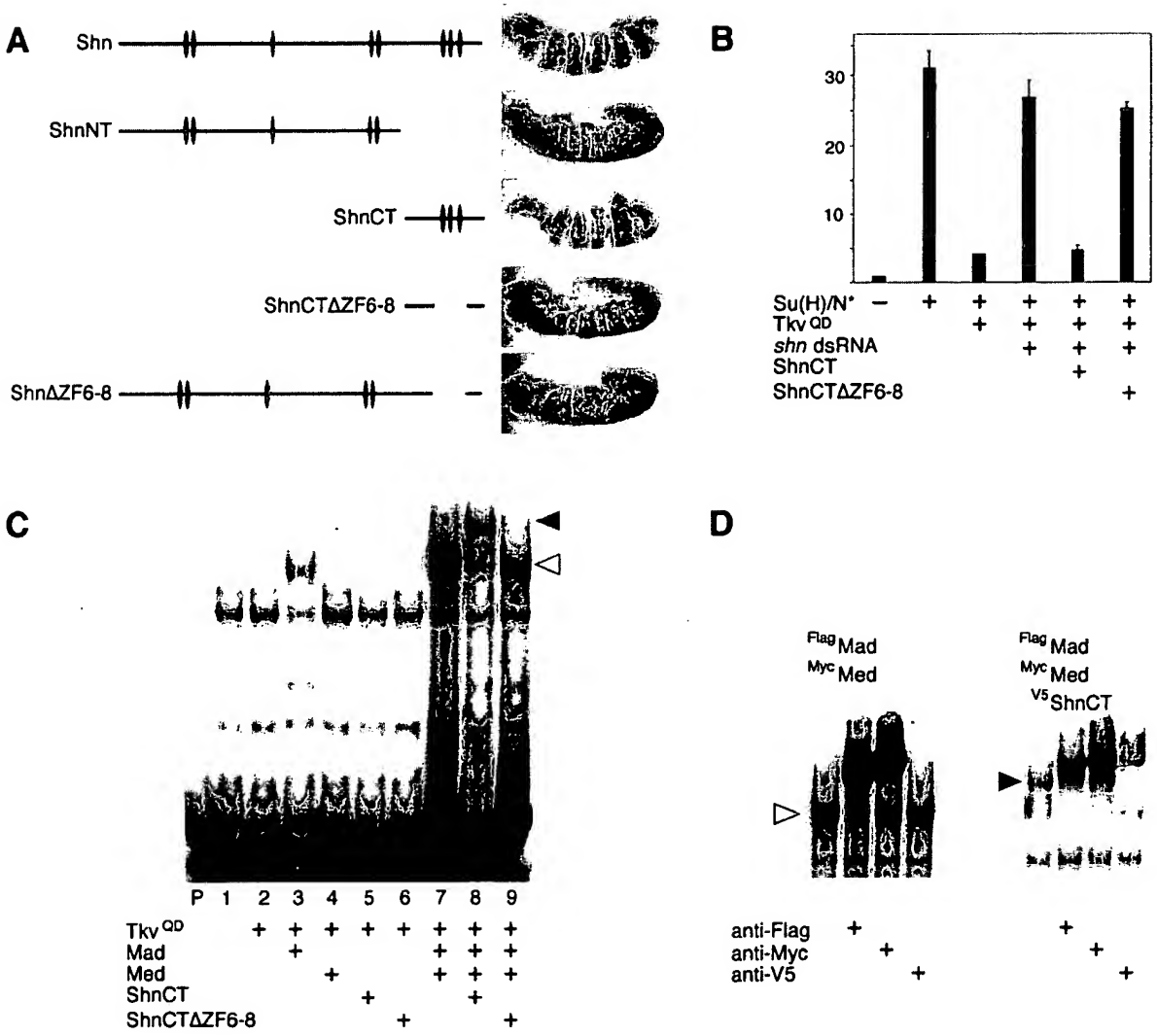


Figure 7. The Carboxy-Terminal Part of Shn Is Both Essential and Sufficient for Dpp-Dependent Repression In Vivo and in Cultured Cells and Forms a Complex with the *brk* Silencer, Mad, and Med

(A) Schematic representations of the Shn derivatives tested are shown to the left (blue ovals indicate zinc finger of the C2H2-type, light blue of the C2HC-type). Embryos transgenic for the illustrated *UAS-shn* constructs were tested for their ability to repress the expression of B14 or *brk* in vivo. The *UAS-shn* constructs were expressed in a shn^{T05} mutant background together with *UAS-dpp* using a *paired-Gal4* driver. β-galactosidase expression of the B14 reporter is shown in stage 14/15 embryos to the right (assay described in Marty et al., 2000). The same results were obtained when these genotypes were assayed for *brk* transcript levels by in situ hybridization (data not shown). Shn, full-length (1–2529); ShnNT, N-terminal portion (1–1888); ShnCT, C-terminal portion (1888–2529); ShnCTΔZF6-8, C-terminal portion lacking zinc fingers 6 to 8 (i.e., residues 2263–2352); ShnΔZF6-8, full-length protein lacking zinc fingers 6 to 8 (i.e., residues 2263–2352). All Shn-fragments contained a Flag-epitope and expression of the tagged proteins was verified by antibody stainings of embryos (data not shown).

(B) S2 cell reporter gene assays. Cells were transfected with the plasmids indicated in combination with *shn* dsRNA to downregulate the expression of the endogenous Shn protein. The loss of Tkv^{QD}-mediated repression caused by *shn* RNAi can by restored by coexpression of ShnCT, but not ShnCTΔZF6-8. Note that the dsRNA used does not affect expression of the transfected carboxy-terminal Shn fragments, since it is derived from an upstream part of the *shn* coding region (corresponding to amino acids 1670–1842). Expression of the Shn construct has no effect on the Notch response as judged by cotransfections with the reporter plasmid containing the Su(H)-response element (data not shown).

(C) Lysates of S2 cells transfected with the indicated plasmids were analyzed in band shift assays using labeled *brk* fragment C as a probe. Transfection of Mad and Med leads to the formation of a protein/DNA complex of slow mobility (lane 7, indicated by the open arrowhead), which can be further retarded by cotransfecting ShnCT (lane 8, filled arrowhead), but not ShnCTΔZF6-8 (lane 9). Note that in single transfections, only Mad has the ability to form a complex with *brk* S (lanes 3–6). Radiolabeled probe S was loaded alone (lane P) and after incubation with extract from untransfected cells (lane 1). The Shn/Mad/Med complex can also be observed in transfection experiments in which no TkvQD is expressed (data not shown), suggesting that phosphorylation of Mad is not a prerequisite for complex formation in vitro.

(D) Lysates from cells expressing ^{Flag}Mad, ^{Myc}Med without ^{V5}ShnCT (left), or with ^{V5}ShnCT (right) in combination with Tkv^{QD} were subjected to band shift assays in the presence of the indicated antibodies. Positions of the Mad/Med-complex (open arrowheads) and Mad/Med/ShnCT-complex (closed arrowheads) are indicated. In the absence of ShnCT, the complex contained both Mad and Med proteins as evidenced by supershifts with both the anti-Flag and the anti-Myc antibodies (Figure 7D, left). In the presence of ^{V5}ShnCT, the low mobility complex was supershifted by antibodies directed against the V5 epitope.

by which Dpp/BMP signaling controls gene expression. Important advances have recently been made by the discovery that a significant aspect of Dpp target gene control involves the repressive action of Brk, whose expression itself is regulated by Dpp (Campbell and Tomlinson, 1999; Jaźwińska et al., 1999a; Minami et al., 1999; Marty et al., 2000). Here, we first confirm and extend these findings by showing that the Dpp signaling system shapes an inverse profile of Brk expression, which serves as a mold for casting the spatial domains of Dpp target genes. Thus, the question of how the Dpp morphogen gradient is converted into transcriptional outputs can be largely reduced to the question of how Dpp generates an inverse transcriptional gradient of *brk* expression. We applied an unbiased approach to this problem by isolating the regulatory elements of *brk*. We then identify and characterize a protein complex that binds to and regulates the activity of these elements in a Dpp dose-dependent manner.

The Two Key Elements of *brk* Regulation

Dissection of the *brk* locus revealed two separable elements with opposite properties: a constitutive enhancer and a morphogen-regulated silencer. Both elements have a direct effect on the level of *brk* expression, and it is the net sum of their opposing forces that dictates the transcriptional activity of *brk* in any given cell. In this sense, expression of the *brk* gene behaves like a spring that is compressed by Dpp signaling. Its silencer and enhancer embody the variable compressing and constant restoring forces, respectively. As stated by Hooke's law, an increased elastic constant (e.g., two copies of the constitutive enhancer) either shifts the *brk* levels toward those normally present at more lateral positions or necessitates a correspondingly higher compressing force (e.g., more silencer elements or higher levels of Dpp signaling). Given the central role Brk plays in controlling growth and pattern together with the direct impact of the two regulatory elements on *brk* levels, it appears inevitable that their quantitative properties must exhibit a fine-tuned evolutionary relationship with each other and with those of the Dpp transduction system. It appears, furthermore, that both the *brk* enhancer as well as the *brk* silencer elements represent ideal substrates for evolutionary changes in morphology.

The Molecular Events at the *brk* Silencer

Based on our combined genetic and biochemical analysis, we propose that upon Dpp signaling the following key players meet at the *brk* silencer elements to execute repression: the Smad proteins Mad and Med and the zinc finger protein Shn. The role of Shn must be to direct the signaling input provided by Mad and Med into transcriptional silencing. In principle, two scenarios can be envisaged by which Shn fulfills this task. Shn could possess repressor activity (presumably via recruitment of corepressors) but lack the ability to bind the *brk* silencer and, hence, depend on Mad/Med for being targeted to its site of action. Alternatively, Shn could be prebound to the silencer, but only be capable of recruiting corepressors upon interaction with Mad/Med. Based on our observation that a Shn/DNA complex cannot be detected in the absence of Mad/Med, we favor the first of these two possibilities. The molecular architecture of the protein complex binding to the *brk* silencer as well as the DNA sequences providing the specificity for the local setup of this complex remain to be determined in detail.

An additional protein, which appears to influence the events at the *brk* silencer, is Brk itself. Genetic experiments indicate that Brk negatively modulates its own expression, forming a short regulatory loop that contributes to the final shape of the Brk gradient (Hasson et al., 2001). This autoregulatory action occurs also via the *brk* silencer element (B.M., unpublished data), suggesting that Brk directly participates in the protein-protein or protein-DNA interactions at this site.

Most regulatory events ascribed to Smad proteins to date concern signaling-induced activation of target gene transcription. In the case of the *brk* silencer Shn could be regarded as a "switch factor" that converts an inherently activating property of Smad proteins into transcriptional repression activity. Indeed, it has been shown that Smad proteins have the ability to recruit general coactivators with histone acetyl transferase activity (reviewed by Massague and Wotton, 2000). However, in an alternative and more general view, Smad proteins per se may provide no bias toward activation or repression. Their main function may be to assemble transcriptional regulatory complexes involving other DNA binding proteins and endow these complexes with additional DNA binding capacity. Such associated DNA binding factors would not only determine target site specificity, but, by their recruitment of either coactivator or corepressor proteins, also define the kind of regulatory influence exerted on nearby promoters (Chen et al., 2002). Since Shn directs Mad/Med activity toward repression, we hypothesize the existence of at least one other such Mad/Med partner in *Drosophila* to account for Mad/Med-mediated activation of gene expression. Such Mad/Med-mediated activation appears to be required for peak levels of *sal* and *vg* transcription (Marty et al., 2000; Campbell and Tomlinson, 1999; Jaźwińska et al., 1999a), as well as for defining gene expression patterns in domains where *brk* expression is completely repressed, e.g., close to the Dpp source of the dorsal embryonic ectoderm (Ashe et al., 2000; Jazwinska et al., 1999b).

The Specificity of Signaling-Regulated Repression

At the heart of our model is the direct causal relationship between the formation of a Shn/Mad/Med/*brk*-silencer complex and the silencing of *brk* gene transcription. Although the two observations have been derived from different experimental data sets (biochemical versus genetic, respectively), there is a firm correlation between the requirements for either event to occur. *brk* is not repressed when either (1) the *brk* silencer elements are lacking or mutated, when (2) Dpp input is prevented (and hence Mad is neither phosphorylated, nor nuclearly localized, nor associated with Med), or when (3) Shn is not present or is deprived of its C-terminal zinc fingers. The same set of requirements was observed for the formation of the Shn/Mad/Med/*brk* complex. Moreover, it is the concurrence of all three of these conditions that appears to provide the exquisite specificity to the Dpp-

regulated silencing of gene transcription. (1) It only occurs in conjunction with a functional *brk* silencer, or an equivalent element. (2) There is an absolute requirement for Dpp input in Shn-mediated silencing. Not even a partial repressor activity of Shn was observed in cells that do not receive Dpp signal (e.g., loss of *shn* function in cells situated in lateral-most positions of the wing disc does not cause a further upregulation of *brk* transcription). (3) Shn represents only one of several zinc finger proteins expressed in Dpp receiving cells, yet none of the other proteins is able to substitute for Dpp-mediated repression. A major determinant for the specificity with which Shn engages in the signaling-dependent protein/DNA complex appears to be the triple zinc-finger motif. Although it is likely that this structural feature is required for contacting specific nucleotides on the *brk* silencer, we can currently not exclude the possibility that some of the zinc fingers mediate protein-protein interactions between Shn and Mad, Med or other cofactors.

While all of the above-discussed elements contribute to the specificity of signaling-regulated repression, it is important to emphasize that one possibility for specificity has not been exploited. The *brk* repression element does not specifically impinge upon the constitutive *brk* enhancer but promiscuously diminishes transcriptional activation by heterologous enhancers. It is likely, therefore, that the *brk* repression element interferes directly with events at the promoter, a property that may permit it to function as a bona fide silencer.

From an Extracellular Gradient to a Nuclear Gradient to Growth and Thresholds

A fundamental characteristic of any morphogen system is that cells at different positions in the concentration gradient respond in qualitatively different ways. Cells must be able to activate different sets of genes at different threshold concentrations. The simplest way by which cells could produce two distinct responses at different threshold concentrations would be the employment of two kinds of receptors of different affinity for the morphogen. This mechanism does not appear to apply for the Dpp morphogen gradient, where Tkv and Punt appear to mediate both low- and high-threshold responses (see Gurdon et al., 1998). Thresholds could also be imposed at any downstream event in the signal transduction cascade. To our surprise, it appears that in the case of the Dpp morphogen, no such gates are in place, and the transcription of the *brk* gene is a negative image of the Dpp gradient. Thus, while our findings provide mechanistic insights into how an extracellular protein gradient is converted into a nuclear gradient of gene activity, they pass the burden of generating threshold effects on to downstream events. Several morphogen gradients operating in the early syncytial embryo, however, have been sufficiently well studied to explain the mechanistic principles of how a gradient of transcriptional activity can specify thresholds of gene activity and tissue differentiation (Struhl et al., 1989; Driever et al., 1989; Struhl et al., 1992; Jiang and Levine, 1993; Hoch and Jäckle, 1993).

A key difference between such embryonic transcriptional gradients and that of *brk* concerns the nature of their outputs: while all of them affect cellular patterns, Brk also controls growth. Flattening the *brk* gradient during development has catastrophic effects: reducing its high end causes overgrowth (Campbell and Tomlinson, 1999), and increasing its low end causes growth arrest (Jaźwińska et al., 1999a; B.M., unpublished data). It may be this fundamental role in growth control that prohibits a discontinuous conversion of the Dpp morphogen gradient into its first transcriptional output. The identification of the elusive growth target(s) controlled by the Brk gradient represents one of the major challenges in the field.

Experimental Procedures

Reporter Transgenes

Inserts of reporter constructs B6 to B38 are derived from genomic lambda phages (G5 and G17, gifts from G. Campbell) and were subcloned into the P element reporter plasmid pX27 (Ségalat et al., 1994). Inserts of B71 to B220 were obtained by PCR, using B14 as a template. Constructs B255 and B261 consist of B216 plus a PCR fragment representing C (B255) or part of C (B261). Constructs B261 to B413 consist of B216 plus a double-stranded oligonucleotide derived from C. The sequence of the C subfragment S is as follows (from distal to proximal): AGTGTCTGGCGGCGTAGCAAGACTGGC GACATTCTGTCTGGTGGCGATCGCC. B413 contains a mutated form of S (mutated bases in lower case): AGTGTCTGGCGGCGTAG CAAGACTGGCGACATTCTtTaTGGTGGCGATCGCC. The insert of D270 is a chimera of constructs 10ΔG and 10-En-mut as shown in Figure 2 of Müller and Basler (2000). For construct DB271, fragment C was inserted at the 5′ position of the *hsp70* promoter of D270. The *eve-stripe-2* enhancer is represented by the MSE construct as published in Small et al. (1992). To obtain EB429, four copies of S were cloned into the EcoRI site of the MSE construct.

Marked Clones of Mutant Cells

Clones of mutant cells were generated by Flp-mediated mitotic recombination, subjecting late second or early third instar larvae to a 35°C heat-shock for 30 min. All mutant alleles used are molecular nulls. Genotypes of dissected larvae were as follows. *Mad* mutant clones: y w hsp70-flp; Mad[12] FRT40/ubi-nlsGFP FRT40; DB271. *Med* mutant clones: y w hsp70-flp; FRT82 e Med[1]/FRT82 2xhsp70-πmyc; DB271. *tkv* mutant clones: y w hsp70-flp; tkv[a12] FRT40/ubi-GFP FRT40; DB271. *shn* mutant clones: y w hsp70-flp; FRT42 shn[TD5]/FRT42 hsp70-GFP; D270 or DB271. *tub>brk* clones: y w omb-lacZ hsp70-flp; tub>CD2,y+>brk and y w hsp70-flp; CyO[sal-lacZ], tub>CD2,y+>brk.

Immunohistochemistry

Imaginal discs from third instar larvae were fixed and stained by standard techniques. Antibodies were rabbit polyclonal anti-β-Gal (Cappel), mouse anti-cMyc (1-9E10.2, Santa Cruz Biotechnology), anti-rabbit 594 Alexa and anti-mouse 488 Alexa fluorescent secondary antibodies (Molecular Probes). To detect β-galactosidase activity, third instar larval discs were fixed and subjected to a standard X-gal color reaction for 2 hr at 37°C. For all X-gal stainings shown in this study, at least four independent transgenic lines were analyzed at standardized reaction conditions (2 hr at 37°C), and a representative disc was chosen for presentation.

S2 Cell Plasmids

S2 cell reporter plasmids containing the *brk* silencer were generated by inserting 100 bp of the 3′ end of fragment C (comprising subfragment S) between the EcoRI and Asp718 sites in hsplacCasper and 4xSuh-lacZ (Kirkpatrick et al., 2001). Epitope-tagged versions of Tkv^{QD}, Mad, Med, ShnCT, and ShnCTΔZF were cloned in the vector pAc5.1B/V5His (Invitrogen) for constitutive expression under the control of the *actin5c* promoter. Plasmids for constitutive expression of Su(H) and activated Notch were a gift from A. Laughon. dsRNA fragments were generated corresponding to nucleotides 658–1230

and 5011–5531 of the *Mad* and *shn* open reading frames, respectively.

Transfections and Reporter Gene Assays

For reporter gene assays 1.5×10^6 S2 cells were transfected with a total of 200 ng of DNA using the Effectene Transfection Reagent (Qiagen) (20 ng reporter plasmid, 5 ng of a plasmid constitutively expressing firefly luciferase, the indicated amount of expression plasmids and pAc5.1B/V5His to bring total DNA to 200 ng). For RNAi experiments 50 ng of the appropriate dsRNA fragment were cotransfected. Cells were lysed 48 hr after transfection for β-galactosidase and luciferase assays.

Band Shift Assays

The ^{32}P-labeled oligonucleotide probe (corresponding to silencer fragment S, see above) was generated by annealing and filling in overlapping oligonucleotides in the presence of [α-^{32}P]ATP. Epitope-tagged proteins were expressed in *Drosophila* S2 cells transfected with 100 ng of each expression plasmid. After 48 hr, cells were lysed in 100 μl of 100 mM TrisHCl (pH 7.8), 0.5% Triton X-100, 1 mM DTT, and protease inhibitors. For mobility shifts, 30 μg of protein was mixed with 10,000–20,000 cpm of probe in Binding Buffer (5×: 25 mM Tris-HCl [pH 7.6], 30% glycerol, 400 mM KCl, 50 mM $MgCl_2$, 50 μM $ZnCl_2$, 0.25% NP-40). Binding was allowed to proceed for 30 min on ice. Protein-DNA complexes were separated from free probe on 4% nondenaturing polyacrylamide gels (at room temperature for 130 min at 160V in 0.5 × TBE). For supershifts, the following antibodies were added to the binding reaction: 20 ng of monoclonal anti-Flag (M2, Sigma), 8 ng of monoclonal anti-Myc (9B11, Cell Signaling), 0.5 μg monoclonal anti-V5 (Invitrogen).

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